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BACE-1 and Caspase-3 AlphaLISA Protease Assays Using Whole-Protein Substrates

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1 Abstract

The cleavage of proteins by intracellular or extracellular proteases results in protein and peptide products that are hallmark biomarkers in many pathologies, such as Alzheimer's and cardiovascular diseases. Therefore, the identification of specific modulators for proteases makes up a significant part of current drug discovery efforts. Here, we describe the application of the AlphaLISA® technology for monitoring protease cleavage activity of whole protein substrates. Using two protease models, β-secretase (BACE-1) and Caspase-3, we show that physiological protein substrates can be cleaved into products that can be directly detected and quantified. Interestingly, both proteases have a higher affinity for these physiological protein substrates compared to short peptides encompassing the enzyme cleavage sites. Known inhibitors of these enzymes generated IC_{50} values in agreement with the literature. Protease assays with whole-proteins offer the advantage of using physiological enzymatic substrates that could uncover novel regulators. In addition, assays developed using full-length substrates are applicable to cell-based measurements, which open new possibilities for better understanding complex protease regulation.

2 Materials and Methods

BACE-1

Cleavage (reagents are diluted in reaction buffer (RB1): 10 mM Sodium Acetate buffer, 1% Triton X-100, pH 4.0):

- 5 µL of Amyloid Precursor Protein α; sAPPα (Sigma S9564)
- 5 µL of β-site of APP cleaving enzyme; BACE-1 (R&D Systems 931-AS-050)

Incubate 60 minutes at 23ºC

Detection (Bead mixes are diluted in 100 mM Hepes buffer, 0.1% Casein, 0.5% Triton X-100, 1 mg/mL Dextran T-500, 0.05% Proclin-300, pH 8.0):

- 20 µL of biotinylated-anti-APP (Covance) [1 nM final] / anti-sAPPβ Acceptor Beads (PerkinElmer) [10 µg/mL final] mix

Incubate 60 minutes at 23ºC

- 20 µL of Streptavidin Donor Beads (PerkinElmer) [40 µg/mL final]

Incubate 30 minutes at 23ºC

Samples were read from a 384-well Optiplate using the EnVision® Multilabel Plate Reader (PerkinElmer).

Caspase-3

Cleavage (reagents are diluted in RB2: 25mM HEPES, 0.1% Casein, 1mg/mL Dextran, 0.5% Triton X-100, 0.05% Proclin-300, 10mM DTT, pH 7.4):

- 5 µL of Poly (ADP-ribose) polymerase; PARP protein (R&D systems 4667-250)
- 5 µL of Cysteinyl aspartate-specific protease-3; Caspase-3 enzyme (R&D systems 707-C3-010)

Incubate for 60 minutes at 37°C

Detection (Bead mixes are diluted in RB2):

- 20 µL of biotinylated-anti-PARP cleavage site (Novus Biologicals) [1 nM final] /anti-PARP (R&D systems) conjugated to Acceptor Beads (PerkinElmer) [10 µg/ml final]

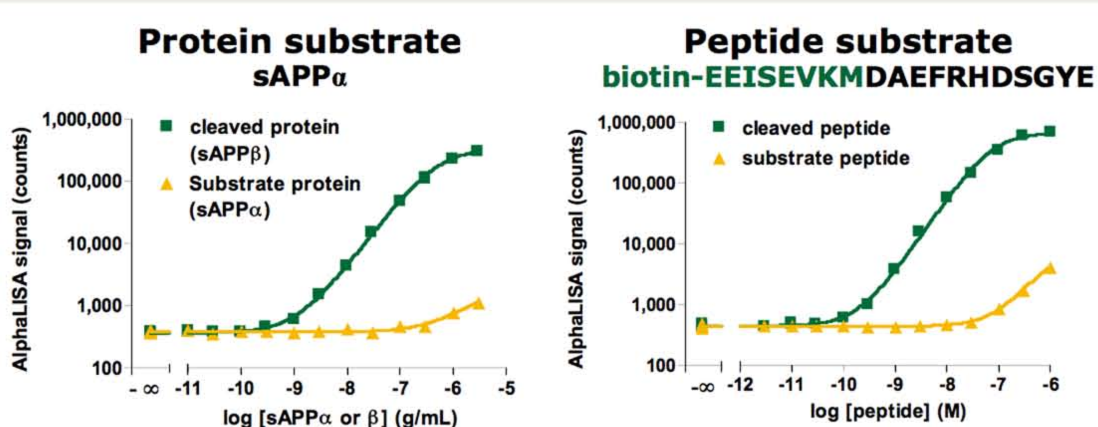
Incubate for 60 minutes at 23°C

- 20 µL of Streptavidin Donor Beads (PerkinElmer) [40 µg/ml final]

Incubate for 30 minutes at 23°C

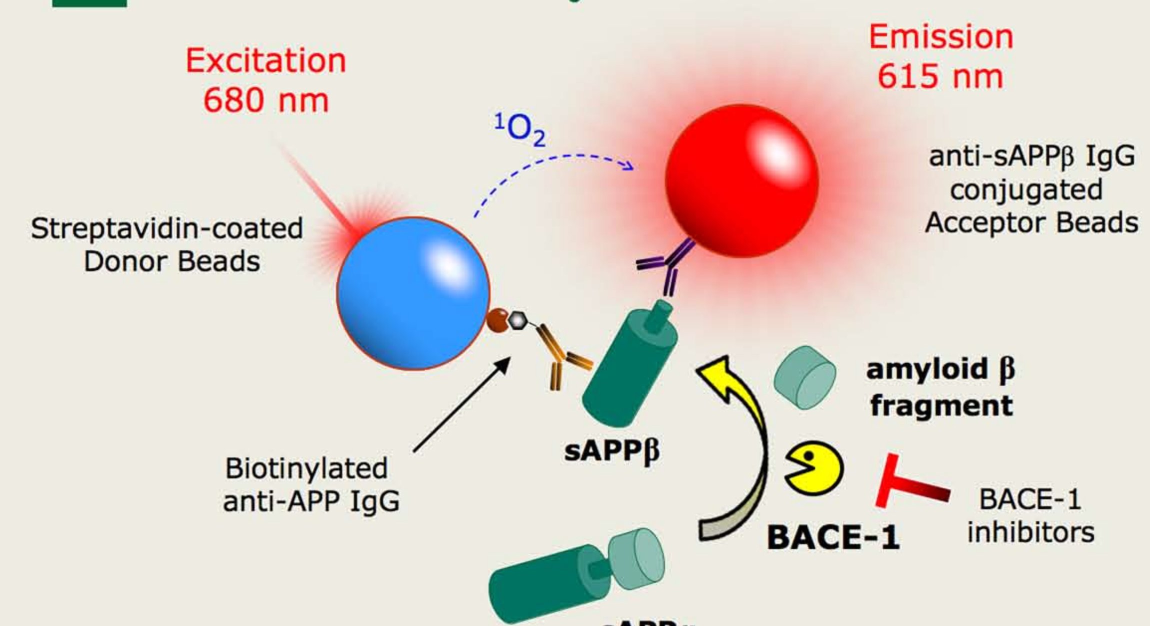
Samples were read from a 384-well Optiplate using the EnVision Multilabel Plate Reader (PerkinElmer).

3 BACE-1 Assay Selectivity



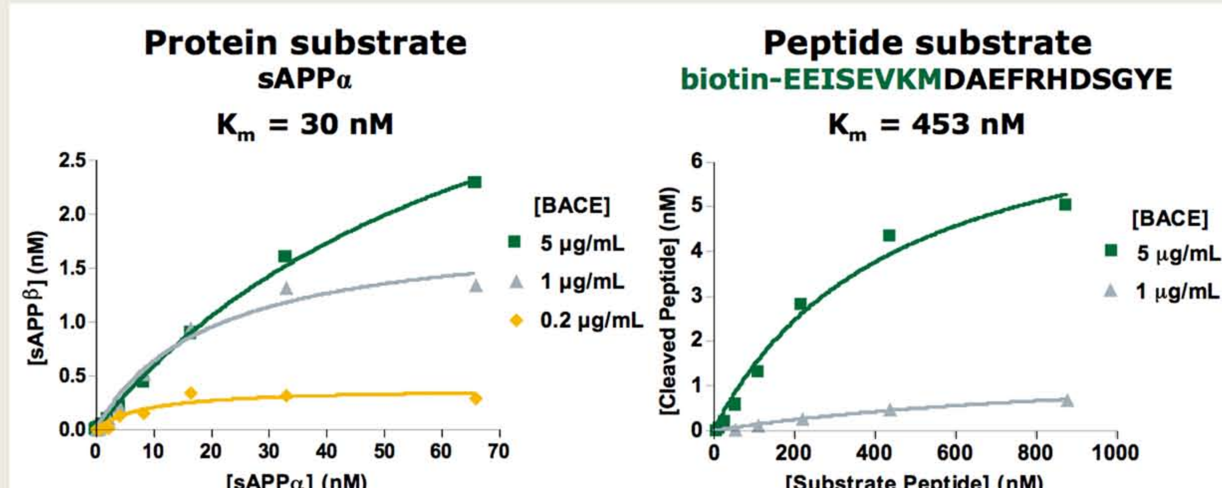
To test specificity we performed titration curves for the substrate and product. Since the affinity of the antibodies for the two proteins (and peptides) differs over three logs, we confirmed that the antibody pair was selective for cleaved forms over substrate forms.

4 BACE-1 Assay



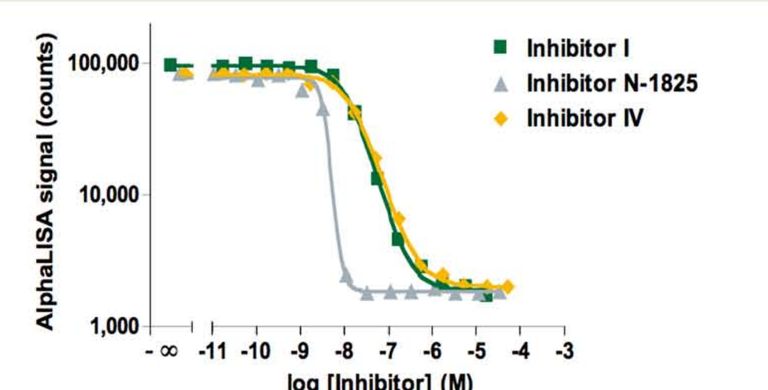
sAPPα is cleaved to sAPPβ by the action of BACE-1. The anti-sAPPβ binds specifically a neo-epitope present on the cleaved protein. The biotinylated anti-APP brings the streptavidin Donor Beads and the antibody-conjugated Acceptor Beads into proximity to generate an AlphaLISA signal. The presence of an enzyme inhibitor blocks its activity and results in a signal decrease. A biotinylated peptide encompassing the cleavage site was also used as substrate for BACE-1, and was detected with the anti-sAPPβ antibody conjugated Acceptor Beads.

5 Cleavage by BACE-1



Substrate sAPPα titrations were performed using three different BACE-1 concentrations. The cleavage reaction was incubated for 60 minutes, followed by addition of AlphaLISA reagents. Results were converted to quantities of sAPPβ by using a standard curve produced in parallel under the same assay conditions.

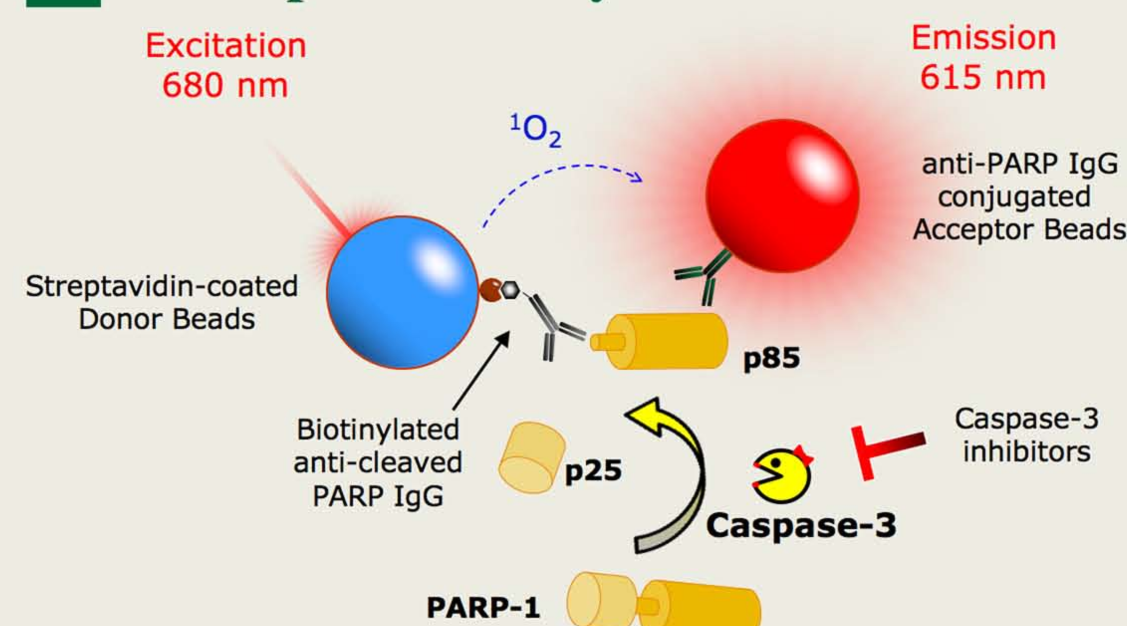
6 Inhibition of BACE-1 Activity



Inhibitor	I	N-1825	IV
S/B	57	48	42
IC_{50} (nM)	14	3.6	18
Literature IC_{50} (nM)	30	0.3	15
Cat#	BioVision 7501-1	Bachem N-1825	Calbiochem 565788

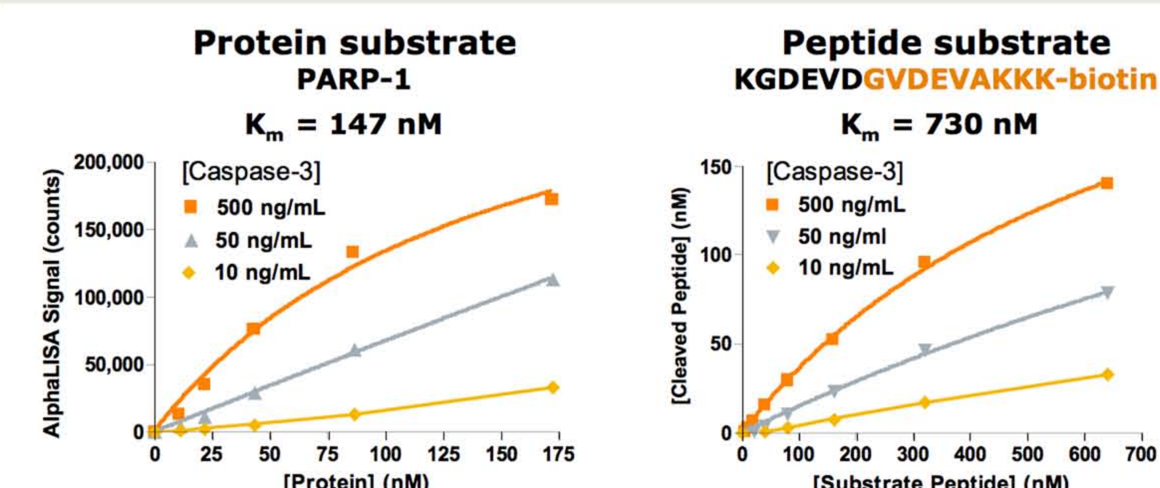
BACE-1 inhibitor I, N-1825, and IV were tested on enzyme activity using 1.0 µg/mL (7.4 nM) BACE-1 and 3.0 µg/mL (30 nM) of sAPPα. Different concentrations of each inhibitor were incubated with BACE-1 at 23°C for 30 minutes, before adding the BACE–inhibitor mixes to sAPPα for the cleavage reaction.

7 Caspase-3 Assay



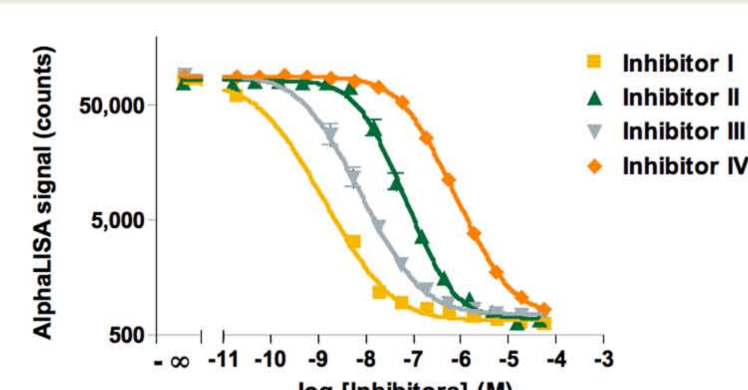
Caspase-3 cleaves PARP-1 to a 85 kDa fragment (p85). A biotinylated antibody binds specifically a neo-epitope present on the cleaved protein p85. The biotinylated anti-p85 brings the streptavidin Donor Beads and the antibody-conjugated Acceptor Beads into proximity to generate an AlphaLISA signal. The presence of an enzyme inhibitor blocks its activity and results in a signal decrease. A biotinylated peptide encompassing the cleavage site was also used as substrate for Caspase-3, and was detected with the Acceptor Beads conjugated p85 specific antibody.

8 Cleavage by Caspase-3



Substrate PARP-1 titrations were performed using three different Caspase-3 concentrations. The cleavage reaction was incubated for 60 minutes at 37°C, followed by addition of AlphaLISA reagents. For peptide substrate, the results were converted to quantities of cleaved peptide by using a standard curve produced in parallel under the same assay conditions.

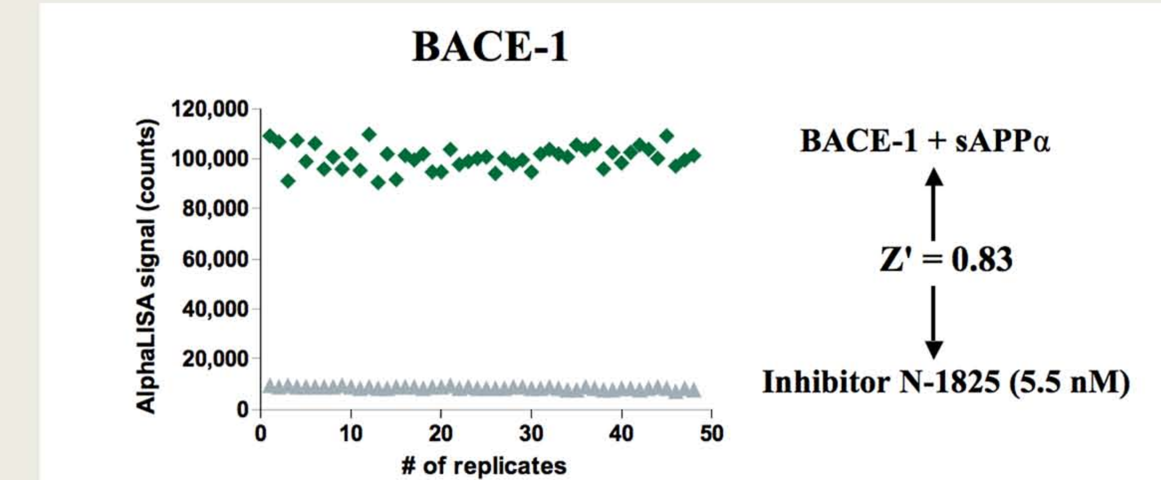
9 Inhibition of Caspase-3 Activity



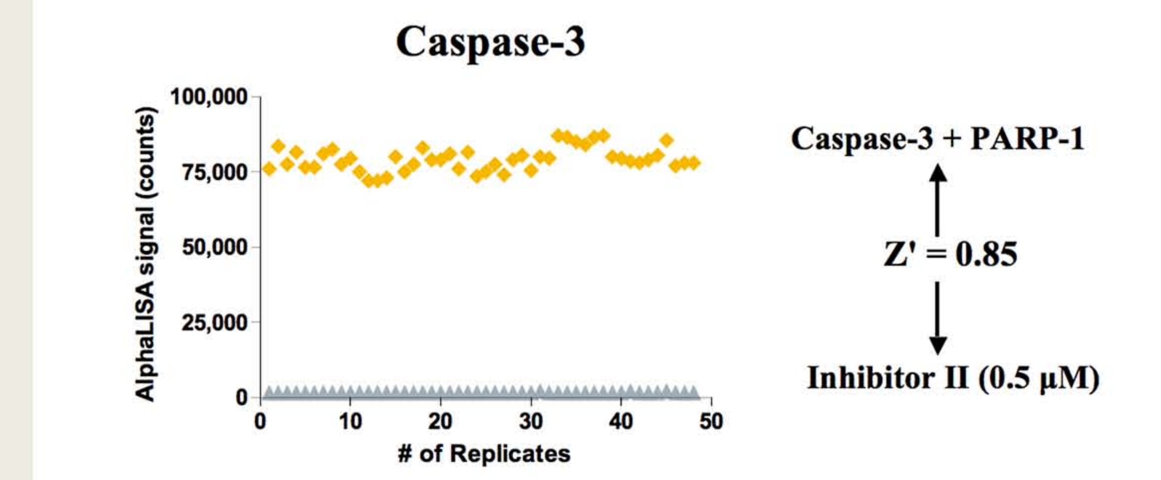
Inhibitor	I	II	III	IV
S/B	137	121	122	111
IC_{50} (nM)	0.09	9.2	0.65	77.4
Literature IC_{50} (nM)	0.2	110	-	193
Cat# (Calbiochem)	235420	264155	218750	235421

Caspase-3 inhibitors I, II, III, and IV were tested on enzyme activity using 62.5 ng/ml (2.2 nM) of Caspase-3 and 20 µg/ml (172 nM) of PARP-1. Different titration curves of inhibitor concentrations were incubated with Caspase-3 for 30 minutes at 23°C, before adding the Caspase-3–inhibitor mixes to PARP-1 for the cleavage reaction.

10 Assay Reproducibility



Plates were loaded with 3 µg/mL of sAPPα, and 1 µg/mL BACE-1 in the presence or the absence of inhibitor. The enzymatic reaction was incubated for 60 minutes at 23°C. The addition of the antibody mix at pH 8.0 stopped the reaction. The Z'-factor values obtained were superior to 0.8 indicating the suitability of the platform for subsequent HTS application. This is a representative result of three independent experiments.



Plates were loaded with 62.5 ng/ml of Caspase-3 and 0.2 µg/ml of PARP-1 in the presence or the absence of inhibitor (0.5 µM inhibitor II pre-incubated 30 minutes at 23°C). The enzymatic reaction was incubated at 37°C for 60 minutes. The Z'-factor values obtained were superior to 0.8 indicating the suitability of the platform for subsequent HTS application. This is a representative result of three independent experiments.

11 Summary

- We developed high throughput assays for monitoring protease cleavage activity on whole protein substrates using AlphaLISA technology.
- BACE-1 and Caspase-3 have higher affinity (lower K_m) for their true protein substrates compared to the tested peptides harbouring the cleavage site. The observed K_m values are also lower than those reported in the literature for fluorogenic peptides (BACE-1: 9 µM. Caspase-3: 9.7 µM).
- Inhibitors of these enzymes resulted in IC_{50} values in agreement with the literature.
- The Z'-factors obtained were superior to 0.8, indicating suitability and robustness of this platform for HTS applications.
- The use of antibodies against the cleavage site neo-epitope allows for signal generation assays.
- The AlphaLISA technology platform is well-suited for compound characterization in monitoring protease activity on physiologically relevant targets.

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